Development and Validation of a Multiplex Add-On Assay for Sepsis Biomarkers Using xMAP Technology

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Background: Sepsis is a common and often fatal disease. Because sepsis can be caused by many different organisms, biomarkers that can aid in diagnosing sepsis and monitoring treatment efficacy are highly warranted. New sepsis markers may provide additional information to complement the currently used markers.

Methods: We used a combination of in-house and commercially available multiplex immunoassays based on Luminex® xMAP technology to assay biomarkers of potential interest in EDTA-plasma samples.

Results: A 3-plex assay for soluble urokinase plasminogen activator receptor (suPAR), soluble triggering receptor expressed on myeloid cells-1 (sTREM-1), and macrophage migration inhibiting factor (MIF) was developed and validated in-house. This 3-plex assay was added to a commercially available interleukin-1/H9252 (IL-1/H9252), IL-6, IL-8, granulocyte/macrophage colony-stimulating factor, and tumor necrosis factor-H9251-human cytokine panel. No cross-reactivity was observed when the assays were combined. Correlation between values obtained with the 8-plex, the 5-cytokine panel, the 3 in-house 1-plex assays, and a suPAR ELISA ranged from 0.86 to 0.99. Mean within- and between-run CVs were 8.0% and 11%, respectively. Recoveries of suPAR, sTREM-1, and MIF calibrators were 108%, 88%, and 51%, respectively. In plasma collected from 10 patients with bacterial sepsis confirmed by blood culture, the assay detected significantly increased concentrations of all 8 analytes compared with healthy controls.

Conclusions: A commercially available xMAP panel can be expanded with markers of interest. The combined multiplex assay can measure the 8 analytes with high reproducibility. The xMAP technology is an appealing tool for assaying conventional cytokines in combination with new markers.

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Sepsis, a common and often fatal disease, is defined as the presence of infection in the context of systemic inflammatory response syndrome (1). To reduce sepsis mortality and morbidity, fast and reliable diagnosis is important; however, the complex pathology of the disease makes this difficult. These facts fueled the search for a reliable sepsis marker. Many potential biomarkers have been investigated, but only C-reactive protein and procalcitonin are used routinely (2–4). The search for a single “magic bullet” marker might be misguided, however; instead of a single marker, a combination of markers might be needed to improve diagnosis, prognosis, treatment efficacy, and ultimately, survival (2).

The majority of studies on new sepsis biomarkers have examined one biomarker at a time. Individual measurements of the plasma concentration of each putative marker incur considerable time, cost, and sample volume, limiting the systematic examination of multiple markers. However, the recently introduced xMAP technology from Luminex allows multiplexing of analytes in solution with flow cytometry. Using a proprietary technique, Luminex internally color-codes xMAP microspheres with 2 fluorescent dyes. With different ratios of these dyes, 100 distinctly colored bead sets are produced, and each bead set can be conjugated with a different capture antibody. The use of R-phycoerythrin–labeled detection antibodies allows quantification of antigen–antibody reactions occurring on the microsphere surface by measurement of the relative fluorescence intensity. The system is potentially capable of measuring 100 different analytes in a single

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Materials and Methods

**Antibodies, Calibrators, Buffers, and Reagents**

MIF and sTREM-1 calibrators and antibodies were purchased from R&D Systems. Calibrators were recombinant human MIF (289-MF-002) and recombinant human TREM-1/Fc Chimera (1278-TR-050). The MIF and sTREM-1 capture antibodies were mouse anti-human monoclonals (MAB289 and MAB1278), and the MIF and sTREM-1 detection antibodies were affinity-purified, biotinylated goat anti-human polyclonals (BAF289 and BAF 1278).

Recombinant human suPAR (residues 1–277) was expressed and purified from culture supernatants of transfected Chinese hamster ovary cells by use of an anti-suPAR antibody column (clones VG-1, V11.5, and V12.3). The suPAR capture antibody was a rat anti-human monoclonal antibody (VG-1) targeting the D3 domain of suPAR. The suPAR detection antibody was an affinity-purified, biotinylated rabbit polyclonal antibody. ViroGates donated the suPAR antibodies. Fluorescently labeled microspheres were obtained from Luminex Corporation.

IL-1β, IL-6, IL-8, GM-CSF, and TNF-α beads, antibodies, antigens, and assay buffers (LHC0003) were purchased from BioSource International. Polyclonal R-phycocerythrin–labeled goat anti-mouse F(ab′)2, immunoglobulins were obtained from DakoCytomation. 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide were purchased from Pierce Biotechnology. MES [2-(N-morpholino)ethanesulfonic acid] phosphate-buffered saline (PBS; 138 mmol/L NaCl, 0.0027 mol/L KCl, 0.01 mol/L phosphate), and containing 1 mL/L Tween 20 were obtained from Sigma-Aldrich.

**Equipment**

The Luminex100 was obtained from Luminex Corporation. STarStation (Ver. 2.0; Applied Cytometry Systems) was used as the acquisition and analysis software.

Washes were performed with 96-well, multiscreen filter plates (MABVN1250), a multiscreen vacuum manifold, and a Chemical Duty Pump (Millipore). During incubation, the plates were placed on a titer plate shaker (Titertek). Liquid handling was performed with calibrated, adjustable, precision pipettes.

**suPAR ELISA**

Maxisorp 96-well plates (Nunc) were incubated overnight at 4°C with 300 ng/well of anti-suPAR clone VG-1 capture antibody. Plates were then washed with PBS containing 1 mL/L Tween 20, blocked 1 h at room temperature with StabilGuard Biomolecule Stabilizer (SurModics), diluted 1 to 1 in PBS, and dried. Samples and calibrators were diluted 1 to 1 in PBS containing 10 g/L BSA and 1 mL/L Tween 20. After the plates had been incubated at 37°C for 1 h and washed 5 times, 100 ng of affinity-purified rabbit anti-suPAR antibody was added to each well. The plates were incubated 1 h at 37°C and washed 5 times, after which 100 ng of horseradish peroxidase–conjugated donkey anti-rabbit IgG antibody (Amersham Biosciences) was added to each well. After
the plates had been incubated for 1 h at 37 °C and washed 10 times, 100 μL of horseradish peroxidase reagent (DY999; R&D Systems) was added to each well. After the plates had been allowed to sit for 15 min at room temperature, the color reaction was stopped by addition of 1 mol/L H₂SO₄ and the absorbance was read at 450 nm. All measurements were performed in duplicate. The suPAR ELISA had within- and between-run CVs of 4.0% and 9.3%, respectively, and the limit of detection (LOD) was calculated to be 63 ng/L.

**PLASMA SAMPLES**

Blood samples were collected from 10 patients who were admitted to Copenhagen University Hospital, Hvidovre, with blood culture-confirmed bacterial sepsis during spring 2005. Whole blood from the patients was drawn on the second day of their hospital stay and placed in a 6-mL dipotassium EDTA-containing Vacutainer Tube (Becton Dickinson). *Pneumococcus pneumonia* and *Escherichia coli* from 4 patients. All patients fulfilled at least 2 of the systemic inflammatory response syndrome diagnostic criteria. Two patients were admitted to the intensive care unit, and the remaining 8 were admitted to the Department of Infectious Diseases. One patient died after 1 week of hospitalization. Donor plasma was drawn from healthy members of the research group and placed in 6-mL dipotassium EDTA-containing Vacutainer Tube (Becton Dickinson). Donor plasma was isolated from the blood of 6 patients and *Escherichia coli* from 4 patients.

**MICROSPHERE CONJUGATION**

Monoclonal antibodies were covalently conjugated to carboxylated Luminex beads according to the procedure suggested by the manufacturer, with minor modifications (17). Briefly, to determine optimal antibody conjugation concentration, we pelleted stock microsphere solutions containing 4 × 10⁶ beads by centrifugation at 13 000g for 2 min, removed the supernatant, and resuspended the beads in distilled H₂O. We repeated the centrifugation, removed the supernatant, and resuspended the beads in 80 μL of 100 mmol/L monobasic sodium phosphate. We then added 10 μL each of 50 g/L N-hydroxysulfosuccinimide and 50 g/L EDC (both diluted in distilled H₂O). After vortex-mixing, the microspheres were incubated for 20 min in the dark at room temperature. The microspheres were then washed twice in 50 mmol/L MES buffer (pH 5.0) and aliquoted into 8 tubes with ~500 000 beads in each tube. To determine the optimal conjugation efficiency, we added 0, 0.5, 1, 2.5, 5, 10, 20, or 40 μg of monoclonal antibody in 500 μL of MES buffer. The coupling reaction was incubated for 2 h at room temperature with rotation. After washing the beads twice with PBS containing 0.5 mL/L Tween 20, we resuspended the beads in PBS containing 10 g/L BSA and 0.5 g/L sodium azide and stored them in the dark at 2–8 °C.

The antibodies were conjugated to the following bead sets: suPAR to bead 33, TREM-1 to bead 38, and MIF to bead 56. We determined the concentration of beads by counting, on the Luminex₁₀₀₀, 0.5 μL of beads diluted 1:400 in PBS containing 10 g/L BSA. We determined the conjugation efficiency by incubating 5000 beads resuspended in PBS containing 10 g/L BSA with 400 ng/well of R-phycocerythrin–conjugated goat anti-mouse F(ab’)₂ immunoglobulins for 30 min. After determining the optimal conjugation concentration, we conjugated a bulk of 5 × 10⁶ beads.

**CALIBRATION CURVE PREPARATION**

The lyophilized MIF and sTREM-1 calibrators were reconstituted in PBS–10 g/L BSA, mixed with the suPAR-containing calibrator to form a 3-component concentrate, and stored in 10-μL aliquots at −80 °C. We prepared calibrators daily by adding 1 mL of assay diluent to the lyophilized IL-1β, IL-6, IL-8, GM-CSF, and TNF-α recombinant human cytokines. The 5-cytokine cocktail was calibrated against the respective National Institute for Biological Standards and Control calibration standards, according to the manufacturer’s specifications. We then added 5 μL of the 3-component calibrator to 445 μL of reconstituted 5-cytokine cocktail. We prepared the 7 individual calibrators by performing 1 to 3 serial dilutions. Calibration curves for each analyte were generated by STarStation software.

**ASSAY PROTOCOL**

We followed the BioSource assay protocol (18) when using the premixed human inflammatory 5-plex alone, but made minor modifications when using the 3-plex alone or when combining the 3- and 5-plexes. Briefly, to each designated, prewetted well on the filter plate, we added bead suspension (5-plex premixed beads and/or 5000 microspheres coated with suPAR, stREM-1, or MIF antibody). The beads were washed twice with wash solution, and incubation buffer was added. Samples (50 μL) were diluted 1 to 1 in assay diluent. Individual calibrators were not further diluted. The plate was incubated for 2 h at room temperature on a titer plate shaker (600 rpm). After 2 washes, 100 μL of a detection antibody cocktail (5-plex premixed antibodies and/or suPAR, stREM-1, or MIF biotinylated antibody) was added per well, and the plate was incubated for 1 h at room temperature on a titer plate shaker. After 2 washes, 100 μL of streptavidin–R-phycocerythrin solution was added to each well. Finally, after incubation for 30 min at room temperature on a titer plate shaker and 3 washes, 100 μL of wash solution was added to each well. The plate was then placed in the XY platform of the Luminex₁₀₀₀. In each
well, a minimum of 100 analyte-specific beads was analyzed for both bead designation and R-phycoerythrin fluorescence.

VALIDATION
On the basis of guidelines from the US Food and Drug Administration, we developed a validation program (19) that entailed full validation of the suPAR, sTREM-1, and MIF multiplex assay and partial validation of the IL-1β, IL-6, IL-8, GM-CSF, and TNF-α assay. The program included assessment of selectivity, linear range, LOD, lower limit of quantification (LLOQ), upper limit of quantification (ULOQ), precision, absolute recovery, freezing/thawing stability, and stability at room temperature.

We evaluated assay selectivity by adding all possible combinations of beads, antigens, and detection antibodies. The highest calibrator (calibrator 7) was used. Because IL-1β, IL-6, IL-8, GM-CSF, and TNF-α were delivered in a premixed mixture, it was only possible to test all 5 cytokines against the add-on assay. We further assessed selectivity by analyzing 6 human EDTA-plasma samples, enriched or not enriched, with twice the LLOQ concentration.

We calculated the LOD by adding 3 SD to the mean median fluorescence intensity (MFI) of 10 blanks. Estimation of the LLOQ for TREM-1 and MIF was based on measurements of 15 plasma samples in the concentration ranges of 120–725 ng/L sTREM-1 and of 178–697 ng/L MIF. It was not possible to establish the LLOQ for suPAR with plasma samples because endogenous suPAR concentrations were well above the predicted LLOQ in all plasma samples; thus, we based the suPAR LLOQ on the median fluorescence intensity (MFI) of 10 blanks. Estimation of the LLOQ for TREM-1 and MIF was based on measurements of 15 plasma samples in the concentration range from 4.1 to 108 ng/L. The ULOQ for all analytes were defined as calibrator 7. The criterion for acceptance of LLOQ and ULOQ values was CVs <20%.

We determined assay linearity by diluting with assay buffer 4 plasma samples prepared by enriching 2 plasmas each from 2 healthy donors. Two plasmas were diluted in a series of eight 2-fold dilutions from 1/2 to 1/256, and the other 2 were first diluted 1/1.5 and thereafter in a series of seven 2-fold dilutions from 1/2 to 1/128.

To assess within- and between-run precision, we prepared 4 validation samples by enriching pooled donor EDTA-plasma with the calibrators. The MIF concentrations in the low validation sample were below the LLOQ; therefore, assessment of MIF assay precision was based on 3 validation samples. The samples were analyzed 3 times over 3 days with 5 repetitive determinations per concentration. The acceptance criterion for precision was a mean CV <15%.

We conducted recovery experiments by enriching single donor plasma samples with the following concentrations of pure calibrator: 0, 46, 139, 417, 1250, and 3750 ng/L suPAR and 0, 188, 563, 1688, 5063, and 15 188 ng/L sTREM-1 and MIF. Enriched and nonenriched samples were run on the same plate in duplicate.

We determined freeze/thaw stability by measuring 3 patient EDTA-plasma samples containing the following concentrations: 2.54, 5.34, and 10.94 µg/L suPAR, 5.74, 14.60, and 39.54 µg/L sTREM-1; and 0.28, 0.37, and 1.20 µg/L MIF. suPAR and MIF occurred naturally in the stated concentrations, whereas the sTREM-1 concentrations were achieved by enriching with recombinant sTREM-1 protein. Each cycle consisted of unassisted thawing at room temperature followed by refreezing for 23 h at −80 °C. We determined the short-term temperature stability in plasma at room temperature by removing 200 µL of EDTA-plasma pooled from 2 patient samples after 0, 0.5, 1, 1.5, 2, 3, 5, 7, and 24 h at room temperature and freezing at −80 °C. The index concentrations were 8.98 µg/L suPAR, 12.82 µg/L sTREM-1, and 2.09 µg/L MIF. The day after freezing, aliquots were thawed and analyzed in duplicate.

STATISTICAL ANALYSIS
Statistical analyses were carried out using SPSS data analysis software (Ver.12.0; SPSS Inc.). Bland–Altman analyses were performed to calculate systematic differences and limits of agreement (20). Ideally, the limits of agreement need to be defined a priori and are considered the maximum width of limits that does not impair clinical decision-making. We arbitrarily defined these limits as ~50%.

The Pearson correlation coefficient (r²) was calculated to determine correlation between values measured by the different assays. The Mann–Whitney test was used for comparing values from patients and donors.

ASSAY DEVELOPMENT
The effect of capture antibody concentration on conjugation efficiency varied. sTREM-1 conjugation efficiency reached a plateau at 5 µg of antibody per 500 000 beads. MIF conjugation efficiency showed a slight decrease when the antibody concentration exceeded 5 µg of antibody per 500 000 beads, whereas suPAR conjugation efficiency increased along the entire interval tested. We therefore chose 5 µg of MIF and sTREM-1 antibodies and 20 µg of suPAR antibody per 500 000 beads for conjugation. MFI values obtained for the chosen suPAR, sTREM-1, and MIF conjugation concentrations were 11 156, 17 598, and 17 978, respectively. MFI values obtained for BioSource IL-1β, IL-6, IL-8, GM-CSF, and TNF-α beads conjugated at unknown antibody concentrations were 21 392, 14 800, 12 999, 14 780, and 24 170, respectively.

Using calibrators 7, 5, and a blank, and testing 6 different antibody concentrations in the interval from 0–8 mg/L, we determined the optimum concentrations of suPAR, sTREM-1, and MIF detection antibodies. The signals for the calibrators plateaued at 2–4 mg/L, whereas the signal for the blank kept increasing. We therefore
chose detection antibody concentrations of 2 mg/L for MIF and sTREM-1 and 4 mg/L for suPAR.

Shown in Fig. 1 is a comparison of calibration curves for the suPAR, sTREM-1, and MIF 3-plex (top); the IL-1β, IL-6, IL-8, GM-CSF, and TNF-α 5-plex (middle); and the 2 assays combined (bottom). The shapes of the different calibration curves did not change when the 3-plex and the 5-plex were combined, but the maximum MFI decreased by a mean of 28%. The best calibration curve fit was for the 5-parameter logistic equation: \( y = a + (d - a)/(1 + x/c)^b/g \), where \( x \) is the concentration; \( y \) is the MFI; \( a \) is the estimated MFI at zero concentration; \( b \) is the slope of the tangent midpoint; \( c \) is the midpoint; \( d \) is the estimated MFI at infinite concentration; and \( g \) is the asymmetry parameter.

**ASSAY VALIDATION**

Evaluation of assay selectivity showed that the background fluorescence values for all 8 beads in the absence of bead-specific antigen were below the LOD and that the assay was able to differentiate between enriched and nonenriched samples for all 8 markers in the 6 samples tested.

The established LLOQ for suPAR, sTREM-1, and MIF are shown in Table 1. The ULOQ for suPAR and sTREM-1 were defined as the calibrator 7 concentrations (22,500 and 91,125 ng/L respectively). However, MIF calibrator 7 could not fulfill the precision criteria (within-run CV was 26%); therefore, calibrator 6 (30,375 ng/L) was used as the ULOQ for MIF.

The linearity of the 3-plex is shown in Fig. 2. The LOD for the commercial panel was calculated to be <3 ng/L for all 5 cytokines. The LLOQ was defined as the concentration of calibrator 1 and the ULOQ as the concentration of calibrator 7. For all 5 cytokines, the precision criteria at both the LLOQ and ULOQ were fulfilled. The mean within- and between-run CVs were 8.0% and 11%, respectively (Table 2).

Mean recoveries of suPAR, sTREM-1, and MIF calibrators added to plasma were 108%, 88%, and 51%, respectively (Table 1). Because of the low MIF recovery, the experiment was repeated with citrate- and EDTA-plasma from 3 donors. The results were the same.

The Pearson correlation coefficient \((r^2)\) between added and measured concentrations of the analytes in enriched samples was 1.00 for all 3 analytes.

As shown in Fig. 3, suPAR concentrations in plasma remained stable for 24 h at room temperature and for at least 5 repeated freeze/thaw cycles, with a measured concentration within 20% of the control. sTREM-1 loss exceeded 20% after 1.5 h at room temperature or 3 freeze/thaw cycles, and MIF loss exceeded 20% after 5 h at room temperature or 1 freeze/thaw cycle.

**ASSAY APPLICATION**

We analyzed EDTA-plasma samples from 10 patients and 10 donors in parallel for all 8 markers, using the three 1-plex assays, the 5-plex, the 8-plex, and a standard suPAR ELISA. When we compared the concentrations of the 8 analytes analyzed as one 8-plex with those measured
by the “stand-alone” assays, the Pearson correlation coefficients ranged from 0.86 to 0.99 (mean, 0.95; Table 1). The MIF values in samples from all 10 donors, sTREM-1 values in samples from 5 donors, IL-8 values in samples from 4 donors, and the TNF-α value in a sample from 1 donor were below the LLOQ and therefore not included in the correlation analysis. The correlation coefficient between suPAR concentrations obtained with the 8-plex assay and the standard suPAR ELISA was 0.95.

The Bland–Altman plots of differences and means of results obtained from the assay showed that the standard deviation of the differences increased with analyte concentration. The values were therefore log-transformed before differences and means were calculated and plotted. The 95% limits of agreement between the 8 analytes measured by the 8-plex and the stand-alone assays were as follows: IL-1β, 93%–138%; IL-6, 99%–121%; IL-8, 103%–131%; GM-CSF, 92%–131%; TNF-α, 104%–144%; suPAR, 84%–125%; sTREM-1, 56%–147%; and MIF, 97%–136%. The 95% limits of agreement between suPAR values measured by the 8-plex and by the ELISA were 99%–140%. The suPAR plots are shown in Fig. 4. The 8-plex assay measured systematically higher concentrations than the 1-plex and 5-plexes and the suPAR ELISA. Because of this result, we conducted another experiment that consisted of testing for cross-reactivity between the analytes in pooled donor EDTA-plasma, using the same approach as for testing cross-reactivity with assay buffer. We saw no cross-reactivity when we used EDTA-plasma.

All 8 markers were significantly increased (P < 0.001) in the 10 patients compared with the 10 healthy donors. Individual concentrations and medians of all 8 markers obtained from the 8-plex assay are plotted in Fig. 5.

**Discussion**

To take full advantage of the xMAP technology, it is important to be able to add new markers to the commercially available panels. In this study, we demonstrate that it is feasible to make an add-on assay work in combination with a commercially available cytokine panel.

The first step is bead–antibody conjugation. Our in-house conjugation efficiency is within the range for the beads in the commercial panel. Others have obtained 10-fold less bound antibody than the commercially available beads (21). Of particular importance is the storage and handling of EDC (21). We were always careful to store EDC under the proper conditions and to have excess EDC in the conjugation solution. Interestingly, we observed a decrease in MIF conjugation efficiency when the antibody concentration exceeded 5 μg per 500 000 beads, whereas suPAR conjugation efficiency was still increasing at 40 μg of antibody per 500 000 beads. Giavadoni (21) experienced a similar decrease in bound antibody when the amount of interferon-γ antibody exceeded 2.5 μg per 500 000 beads. The rather high concentration of suPAR antibodies required to achieve acceptable conjugation probably the result of insufficient removal of azide from the antibody solution, which has been reported to influence conjugation efficiency (22).

Research groups have used different protocols for validating their Luminex assays (21–28). We followed the minimal criteria outlined in the Food and Drug Administration guidance document for industry on bioanalytical method validation (19), with the exceptions that we were not able to obtain suitable analyte-free plasma and that we chose to use only 4 validation samples covering the working range instead of the 5 suggested in the guidance document. The analytical sensitivity and precision were comparable to those reported by others (21–28).

Recoveries of suPAR and sTREM-1 were in the expected range, but the mean MIF recovery of 51% in both EDTA- and citrate-plasma was lower than expected. It is well established that cytokines interact with many plasma proteins that may interfere with antibody binding, including heterophilic antibodies, serum-binding proteins, and soluble and membrane-bound receptors. The low recov-

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**Table 1. Characteristics of the 8-plex xMAP assay for human EDTA-plasma.**

| Analyte | LOD, ng/L | LLOQ, ng/L | ULOQ, ng/L | Recovery, % | NIBSC® calibration (1 ng = 1 IU) | Pearson r²  
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<tbody>
<tr>
<td>suPAR</td>
<td>7.7</td>
<td>17</td>
<td>22 500</td>
<td>108</td>
<td>ND*</td>
<td>0.99</td>
<td>0.95</td>
<td></td>
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<tr>
<td>sTREM-1</td>
<td>58</td>
<td>271</td>
<td>91 125</td>
<td>88</td>
<td>ND</td>
<td>0.88</td>
<td>ND</td>
<td></td>
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<tr>
<td>MIF</td>
<td>14.0</td>
<td>105</td>
<td>30 375</td>
<td>51</td>
<td>ND</td>
<td>0.86</td>
<td>ND</td>
<td></td>
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<tr>
<td>IL-1β</td>
<td>&lt;3.0</td>
<td>9</td>
<td>6810</td>
<td>90**</td>
<td>74a</td>
<td>0.97</td>
<td>0.97a</td>
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<tr>
<td>IL-6</td>
<td>&lt;3.0</td>
<td>7</td>
<td>5200</td>
<td>91d</td>
<td>505a</td>
<td>0.98</td>
<td>0.97a</td>
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<tr>
<td>IL-8</td>
<td>&lt;3.0</td>
<td>8</td>
<td>6330</td>
<td>96d</td>
<td>1.1d</td>
<td>0.98</td>
<td>0.97d</td>
<td></td>
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<tr>
<td>GM-CSF</td>
<td>&lt;3.0</td>
<td>13</td>
<td>9940</td>
<td>ND</td>
<td>9a</td>
<td>0.98</td>
<td>0.99d</td>
<td></td>
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<tr>
<td>TNF-α</td>
<td>&lt;3.0</td>
<td>15</td>
<td>11 390</td>
<td>89d</td>
<td>16.4d</td>
<td>0.98</td>
<td>0.93d</td>
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* LOD was calculated by adding 3 SD to the mean of 10 blanks. Acceptance criterion for LLOQ and ULOQ was a CV <20%. To determine recovery, 1 sample was enriched with 5 different concentrations of pure calibrator. Correlations between the different assays were determined by analyzing 20 human plasma samples in parallel.

**a** NIBSC, National Institute for Biological Standards and Control.

**b** CV calculation; SD/mean × 100%.

**c** Results supplied by BioSource.

**d** ND, not determined.
ery of MIF may be a result of its ability to bind human albumin (29). A recent study demonstrated that a MIF ELISA, using the same commercially available antibody combination that we used, detected <1% of the total serum MIF detected by Western blot (30). The same study showed that use of diluent buffers that included BSA led to MIF serum immunoassay interference. Lui et al. (26) studied recoveries of MCP-1 from human serum, using Luminex assays from 3 different manufacturers and a standard ELISA. Mean recovery was ~55% with the Luminex system, whereas ELISA gave a mean recovery ~100% (26). This low recovery with the Luminex system could be, in part, a result of a typical matrix effect, which might be avoided by further dilution of plasma samples as is often done in ELISAs. Multiplexing is a balance between finding the right dilution factor for a single analyte and an acceptable universal dilution factor for multiple analytes.

Although we did not observe any cross-reactivity among the 8 analytes, either when the pure recombinant proteins were added in different combinations or when we combined the 8 calibration curves, the determined concentrations of all 8 analytes were higher when analyzed as an 8-plex vs the stand-alone assays. It was even possible for the 8-plex to quantify sTREM-1 in samples unquantifiable by the 1-plex. This raises the question of the true concentration of sTREM-1 in plasma. To our knowledge, 4 studies have quantified sTREM-1 in plasma/serum (7, 22, 31, 32). There is a 400-fold difference in the LOD among the assays used in the 4 studies, but this difference did not influence their abilities to quantify sTREM-1 in serum samples. The assays used the same primary sTREM-1 antibody but different analytical methods (immunoblot, ELISA, and Luminex technology).

A reference assay is needed, but such an assay is lacking even for cytokines known and analyzed for years (28, 33). In the 8-plex assay, the cytokine values measured in plasma from donors and patients were higher than we expected, especially for IL-1β, TNF-α, and GM-CSF. We analyzed potassium EDTA–plasma several times with the BioSource panel without any add-ons, and the results were consistent. According to the manufacturer, the EDTA-plasma validation experiments were performed with sodium EDTA. Because of this difference, BioSource is currently investigating the correlation between Luminex and standard ELISA measurements using potassium EDTA–plasma samples. A study comparing Luminex panels from different manufacturers found that the BioSource IL-1β multiplex assay measured higher values than the other panels tested when samples were analyzed in parallel (25), and newly published results showed up to a 12-fold difference in determined cytokine concentrations between 2 commercially available Luminex panels (24). Nevertheless, the relative differences in IL-1β, IL-6, and TNF-α between sepsis patients and controls observed in our study are comparable to the relative differences found in an earlier study (34).

Fig. 2. Linearity of the suPAR (top), sTREM-1 (middle), and MIF (bottom) assays.

Dilution tests using 4 plasma samples. For sTREM-1 and MIF, only 3 plasma samples are shown because in one of the plasma samples, these 2 analytes were measurable only in the first 2 dilutions. The y axis shows the analyte concentration as determined with the 3-plex.
We have not found any multiplex studies that report changes in measured analyte concentrations when combining different assays. Further examination of the mechanisms behind these changes exceeded the scope of this study. One might speculate that cross-reactions between the different antibodies included in the assay are a factor.

Table 2. Concentrations of analytes in quality-control samples and within-/between-assay CVs.*

<table>
<thead>
<tr>
<th>Analyte</th>
<th>IL-1β</th>
<th>IL-6</th>
<th>IL-8</th>
<th>GM-CSF</th>
<th>TNF-α</th>
<th>SuPAR</th>
<th>sTREM-1</th>
<th>MIF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>ng/L</td>
<td>CV, %</td>
<td>ng/L</td>
<td>CV, %</td>
<td>ng/L</td>
<td>CV, %</td>
<td>ng/L</td>
<td>CV, %</td>
</tr>
<tr>
<td>1</td>
<td>42</td>
<td>19/32.6</td>
<td>15</td>
<td>17/6.7</td>
<td>13</td>
<td>14/24</td>
<td>91</td>
<td>11/28</td>
</tr>
<tr>
<td>2</td>
<td>107</td>
<td>2.7/6.2</td>
<td>50</td>
<td>6.3/5.3</td>
<td>54</td>
<td>4.9/4.7</td>
<td>147</td>
<td>9.9/13</td>
</tr>
<tr>
<td>3</td>
<td>239</td>
<td>3.6/5.2</td>
<td>121</td>
<td>6.8/8.9</td>
<td>132</td>
<td>8.1/2.4</td>
<td>279</td>
<td>5.7/9.7</td>
</tr>
<tr>
<td>4</td>
<td>2777</td>
<td>3.3/9.8</td>
<td>1290</td>
<td>5.1/8.3</td>
<td>1368</td>
<td>6.2/10</td>
<td>2681</td>
<td>2.3/7.4</td>
</tr>
<tr>
<td>Mean</td>
<td>7.1</td>
<td>13/7.3</td>
<td>8.4</td>
<td>10/4.1</td>
<td>7.2</td>
<td>15/5.3</td>
<td>8.7</td>
<td>8.9/3.6</td>
</tr>
</tbody>
</table>

* The concentrations given are the mean of the concentrations measured in the validation samples. The CVs are reported as the within-assay/between-assay CVs for each validation sample and the mean CV of the 4 samples. CV calculation: SD/mean × 100%.

b NA, not applicable.

Fig. 3. Stabilities of suPAR, sTREM-1, and MIF at room temperature (A), and effects of repeated freezing/thawing on measured suPAR, sTREM-1, and MIF concentrations (B).

Fig. 4. Bland–Altman analysis showing agreement between log-transformed suPAR values obtained from the Luminex 8-plex and the suPAR ELISA (A) or 1-plex (B).

Solid lines are mean values; dotted lines are 2 SD.

We have not found any multiplex studies that report changes in measured analyte concentrations when combining different assays. Further examination of the mechanisms behind these changes exceeded the scope of this study. One might speculate that cross-reactions between the different antibodies included in the assay are a factor,
although we observed no cross-reactivity between the assay components when tested in assay buffer or in plasma. The naturally occurring analytes might act immunogenically differently from the recombinant proteins, and many plasma proteins likely interact with the analytes. These complications make it impossible to compare exact analyte concentrations obtained from the different combinations of assays. Nevertheless, a mean correlation coefficient of 0.95 is impressive and allows comparison of relative changes within and between groups rather than exact concentration differences.

Analyte stability is another important issue to assess during validation. Earlier studies of suPAR stability and MIF half-life yielded results similar to the ones presented here (35, 36). To our knowledge, there are no earlier assessments of sTREM-1 stability or MIF freezing/thawing stability. Short-term storage stability under conditions other than the ones investigated in this study (e.g., in whole blood and at different temperatures) and long-term storage stability need to be evaluated. Our conclusions based on the storage experiments is that the suPAR assay is very robust to differences in sample handling, whereas handling procedures need to be rigorously homogeneous to obtain reliable results for sTREM-1 and MIF. However, it is important to note that stability assessments are specific for the antibody pairs tested and should therefore accompany validations of new assay components (28).

Some of the advantages of multiplexing compared with measuring the same analytes by traditional ELISA are a reduction in pipetting error; a reduction in hands-on time and, therefore, cost; and improved quality of results because freezing/thawing would typically be required for the measurement of multiple analytes by ELISA. Another advantage is the reduced amount of sample needed, which is of particular importance in children, from whom small amounts of plasma are usually obtained, and in critically ill sepsis patients, for whom there is a need for monitoring immune status at several time points. In addition to the immune markers described here, it is possible to add on beads detecting other types of proteins, such as diagnostic molecules (e.g., viral proteins), therapeutic drugs, and human antibodies.

In conclusion, there are pitfalls when using either commercially available panels or in-house–developed assays. Several issues require further study, such as the presence of interfering heterophilic antibodies and other plasma proteins that might skew results, optimization of the pairing of capture and detection antibodies, the best working buffer systems, and the ideal matrix for preparation of calibrators. When combining 2 assays, laboratorians should consider that results are not always directly comparable to those obtained from the 2 individual assays. Therefore, the same assay should be used to analyze all samples in a clinical study.

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References


